DAP-KINASE RELATED PROTEIN

CROSS-REFERENCE TO RELATED APPLICATIONS

5   The present application is related to and hereby incorporates by reference the entire contents of each of application serial no. 08/810,712, filed March 3, 1997, and application serial no. 08/631,097, filed April 12, 1996, the latter of which has been published as PCT publication WO 95/10630 on April 20, 1995. The present application also claims priority from provisional application 60/089,294, filed June 15, 1998, the entire contents of which are hereby

10   incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

          The present invention is directed to a DAP-kinase related protein.

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Description of the Related Art

          One of the factors which determines the proliferation state of cells is the balance between the growth-promoting effects of proto-oncogenes and the growth-constraining effects of tumor-suppressor genes. One mechanism by which

20   tumor-suppressor genes exert their growth-constraining effect is by inducing a cell to undergo a physiological type of death. Such a controlled cell death is evident in a multitude of physiological conditions including metamorphosis, synaptogenesis of neurons, death of lymphocytes during receptor repertoire selection, and controlled homeostasis in the bone marrow and other proliferative tissues, etc. This cell death is regulated by the interaction of

25   the cell with other cells or with cell products, for example through the activity of suitable cytokines.

          Growth-inhibiting cytokines have a double effect on the target cell. They can either inhibit the proliferation of the cell and/or give rise to cell death. To date, blockage or activation of expression of known tumor-suppressor genes was shown to counteract or

30   enhance, respectively, cytokines inhibition of cells growth (Kimchi, 1992) but did not have any effect on the death- promoting action of cytokines. For example, the growth inhibitory response to cytokines, such as TGF-β, was markedly reduced by the inactivation of the Rb gene, or the response to IL-6 was enhanced by introducing activated p53 genes (Pietenpol et al,, 1990; Levy et al, 1993).

Apoptosis is a genetically controlled cell death process which is important in various developmental stages, as well as for cell maintenance and tissue homeostasis (Jacobson et al., 1997). During the last few years, many of the key players in this process have been identified, including receptors, adaptor proteins, proteases, and other positive and

5   negative regulators (Green et al., 1998; White, 1996). One of the positive mediators of apoptosis, which has been cloned by the present inventors, is DAP-kinase (Deiss et al., 1995). This protein was discovered by a functional approach to gene cloning, based on transfections of mammalian cells with anti-sense cDNA libraries and subsequent isolation of death-protective cDNA fragments (Deiss et al., 1995; Deiss et al., 1991; Kimchi, 1998; Kissil

10   et al., 1998; Levy-Strumpf et al., 1998). The anti-sense cDNA of DAP-kinase protected HeLa cells from interferon-gamma-induced cell death, and this property served as the basis for its selection.

DAP-kinase is a calcium/calmodulin-regulated 160 kDa serine/threonine protein kinase associated with actin microfilaments (Deiss et al., 1995; Cohen et al., 1997). Its

15   structure contains at least two additional domains that might mediate interactions with other proteins: ankyrin repeats, and a typical death domain located at the C-terminal part of the protein (Deiss et al., 1995; Cohen et al., 1997). Overexpression of DAP- kinase in various cell lines results in cell death, and this death-promoting effect of DAP-kinase depends on at least three features: the catalytic activity, presence of the death domain, and the correct intracellular

20   localization (Cohen et al., 1997; Cohen et al., 1999). Several independent lines of evidence proved that DAP-kinase is involved in apoptosis triggered by different external signals, including interferon-γ, TNF-α, activated Fas receptors, and detachment of cells from the extracellular matrix (Deiss et al., 1995; Cohen et al., 1997; Cohen et al., 1999; Inbal et al., 1997). A tumor suppressive function was recently attributed to the DAP-Kinase, coupling the

25   control of apoptosis to metastasis (Inbal et al., 1997).

So far, only a few serine/threonine kinases were implicated in the regulation of programmed cell death, either as death- promoting and death-protecting proteins (Anderson, 1997; Bokoch, 1998). One such candidate is the JNK/SAPK (Basu et al., 1998). In one example, it was shown to mediate apoptosis induced by detachment from extracellular matrix

30   (named anoikis) (Cardone et al, 1997). In this system, the JNK pathway is activated by MEKK-1, whose kinase activity is stimulated by caspase cleavage (Cardone et al., 1997). JNK

may antagonize BCL-2 anti-apoptotic effects by phosphorylation (Park et al., 1997; Maundrell et al., 1997).

Another serine/threonine kinase is RIP, which like DAP-Kinase also possesses the death domain. RIP was shown to positively mediate apoptosis in cell cultures (Stanger et al., 1995). However, *in vivo* studies in RIP-deficient mice demonstrated its ability to exert anti-apoptotic effects by mediating the TNF-α- induced TNF-β activation (Kelliher et al., 1998). Other RIP members, RIP2 and RIP 3 were also recently identified and shown to possess pro-apoptotic effects (McCarthy et al., 1998; Sun et al., 1998; Yu et al., 1999).

Among the negative regulators of apoptosis is the protein kinase AKT. This protein was shown to phosphorylate BAD and thereby to prevent it from complexing and blocking the anti- apoptotic activity of BCL-X_L (Datta et al, 1997; del Peso et al., 1997). AKT was also recently shown to phosphorylate pro-caspase-9, thus blocking its normal processing (Cardone et al., 1998).

Recently, the isolation and characterization of novel kinase members, homologous in their catalytic domains to DAP- kinase, was reported (Kawai et al., 1998; Kogel et al., 1998; Sanjo et al., 1998). One protein, named ZIP-kinase, was found to be 80% identical to DAP-kinase within the kinase domain, yet it lacks the CaM-regulatory domain and the other domains and motifs characteristic of DAP-kinase. Zip-kinase contains a leucine zipper domain at the C-terminus and is localized to the nucleus (Kawai et al., 1998; Kogel et al. 1998). The activation of ZIP kinase occurs by a different mechanism involving homo-dimerization, mediated by its leucine zipper domain. However, unlike DAP-kinase, ZIP-kinase is a nuclear protein, which instead of being regulated by a calmodulin-binding domain, is activated by homo-dimerization of its leucine-zipper motifs (Kogel et al., 1998). Another two less conserved nuclear proteins, DRAK1 and DRAK2, which are closely related to each other, and which share 50% identity with the kinase domain of DAP-kinase, were also recently characterized. Like ZIP-kinase, the DRAK1 and DRAK2 proteins also lack the CaM-regulatory domain. The overexpression of these two proteins in NIH3T3 cells induces some morphological changes associated with apoptosis, dependent on the functionality of their kinase domain (Sanjo et al., 1998). Together these kinases form a novel subfamily of serine/threonine kinases, as is evident from multiple sequence and phylogenetic analysis (Inbal et al., 1999).

Ectopic expression of the three wild type kinases, but not their catalytically inactive mutants, induced morphological changes characteristic of apoptosis (Kawai et al., 1998; Sanjo et al., 1998). Yet, in the case of ZIP-Kinase, these results are still controversial (Kogel et al., 1998).

5   Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to the content or a date of any document is based on information available to the applicant at the time of filing and does not constitute an admission as to the correctness of such a statement

SUMMARY OF THE INVENTION

A new protein, DAP-Kinase-related 1 protein (DRP-1), which is a novel homologue of DAP-kinase, has been isolated. This novel calmodulin-dependent kinase is a 42kDa serine/threonine kinase which shows a high degree of homology to DAP-kinase both in its catalytic domain and its calmodulin-regulatory region. The catalytic domain of DRP-1 is also homologous to recently identified ZIP-kinase and, to a lesser extent, to the catalytic domains of DRAK1/2.

DRP-1 is localized to the cytoplasm as shown by immunostaining and cellular fractionation assays. *In vitro* kinase assays indicate that wild type DRP-1, but not a kinase inactive mutant, undergoes autophosphorylation and phosphorylates an external substrate in a Ca2+/CaM-dependent manner. Ectopically expressed DRP-1 is able to induce apoptosis in various types of cells; with this killing being dependent on its kinase activity. The dominant negative form of DAP-Kinase (DAPk DD) is a potent blocker of apoptosis induced DRP-1. Thus, DRP-1 may be a death-promoting protein functioning in the biochemical pathway which involves DAP (death-associated protein)-kinase (e.g., forming a cascade of sequential kinases, one directly activating the other). Alternatively, the two kinases may operate to promote cell death in parallel pathways.

The present invention provides for a DRP-1 protein and functional homologues thereof having at least 85% sequence identity to the DRP-1 sequence of SEQ ID NO:2. Also provided is a fragment of DRP-1, which either is capable of inducing cell death or lacks such capability but instead is capable of inhibiting the activity of DRP-1 or a functional homologue

thereof to induce cell death, and a homologous fragment which has at least 85% sequence identity thereto and which has the same properties.

The present invention further provides an isolated DNA molecule encoding for such DRP-1 protein, functional homologues thereof, or fragments thereof. Also included within the scope of the present invention are isolated DNA molecules which hybridize to the nucleotide sequence encoding DRP-1 protein under moderately or highly stringent conditions and encode a calmodulin-dependent serine/threonine kinase having the property of being capable of inducing cell death.

Other further aspects of the present invention include a composition comprising the DRP-1 protein, functional homologues and fragments thereof, and an antibody which specifically recognizes DRP-1 but does not cross-react with DAP kinase or ZIP kinase.

Yet another aspect of the present invention is directed to a single stranded RNA molecule complementary to at least a portion of the mRNA encoding the DRP-1 protein of SEQ ID NO:2. This single stranded antisense RNA molecule can be used in a method of neutralizing DRP-1 mRNA by hybridizing to the DRP-1 mRNA to prevent its translation into DRP-1 protein.

The present invention also provides a method for screening individuals for predisposition to cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequence of the DAP-kinase homologue, DRP-1. The initiation (ATG) and stop (TAA) codons are boxed. The polyadenylation signal (ATTAAA) is underlined. The kinase domain and the calmodulin regulatory regions are in bold or underlined by a dash, respectively.

Figures 2A-2B show the multiple sequence alignment of the serine/threonine kinase domains (Fig. 2A) of the DAP-kinase-related proteins, DAP-kinase (SEQ ID NO:3), ZIP-kinase (SEQ ID NO:4), DRP-1 (corresponding to residues 13-275 of SEQ ID NO:2), DRAK1 (SEQ ID NO:5) and DRAK 2 (SEQ ID NO:6), conducted according to Hanks and Quinn (1991) with identical amino acids boxed and homologous amino acids shown with gray shading, and the multiple sequence alignment of the calmodulin regulatory regions (Fig. 2B) of DAP-kinase (SEQ ID NO:7), DRP-1 (corresponding to residues 292 to 320 of SEQ ID NO:2), smMLCK (SEQ ID NO:8), CaMKIIa (SEQ ID NO:9), CaMKI (SEQ ID NO:10),

CaMKIV (SEQ ID NO:11), and ZIP-Kinase (SEQ ID NO:12) conducted manually, keeping the conserved (boxed) regions aligned to each other. The corresponding region of ZIP-Kinase which does not contain homology to DAP-Kinase and ZIP-Kinase CAM-regulatory regions is given at the bottom of Fig. 2B.

5   Figure 3A shows Northern blot analysis of polyA+RNA extracted from various cell lines for mRNA expression of DRP-1, Figure 3B show Western blot analysis of *in vitro* transcription and translation of DRP-1, and Figure 3C shows protein expression of DRP-1 in HeLA cells on an immunoblot.

Figures 4A and 4B show control COS-7 cells and cellular localization of

10   DRP-1 in COS-7 cells, respectively, and Figure 4C shows a Western blot of fractions from a detergent extraction of COS-7 cells transfected with a pCDNA3 vector expressing either FLAG-tagged DRP-1 or DAP-Kinase.

Figure 5A shows *in vitro* kinase activity of DRP-1 and Figure 5B shows a Western blot of DRP-1 proteins.

15   Figures 6A-6B show fluorescent microscope images of 293 cells transfected by pCDNA3-luciferase as a negative control (Fig. 6A), by pCDNA3-ΔCaM DAP-Kinase as positive control (Fig. 6B), by pCDNA -DRP-1 (Fig. 6C), and by pCDNA3-K42A DRP-1 (Fig. 6D). Apoptotic cells are indicated by arrows.

Figure 7 shows the scores of apoptotic cells in a graph of the percentage of

20   apoptotic cells resulting from the transfections of Figs. 6A-6D.

Figures 8A and 8B show DRP-1 protein expression in 293 transfected cells in immunoblots to anti-FLAG antibodies (Fig. 8A) and anti-vinculin antibodies (Fig. 8B).

Figure 9A shows that DAP kinase death domain protects from DRP-1 induced apoptosis, and Figure 9B shows an immunoblot of DRP-1 protein expression in 293

25   transfected cells.

Figure 10A shows a schematic representation of a series of generated deletion mutant, and Figure 10B shows an immunoblot containing extracts of 293 cells transiently transfected with GFP and the series of deletion mutants, (DRP-1 fragments, cloned in pCDNA3, and tagged with HA epitope at the C-terminus), as in Figs. 8A and 8B are probed

30   with anti-HA antibodies for DRP-1 detection and anti-vinculin antibodies to quantitate the loaded protein amounts. In Figure 10B, pCDNA3^2-luciferase is the negative control.

Figure 11A shows fluorescent microscope images of the transiently transfected cells of Fig. 10B, and Figure 11B shows a graph of the score in percent apoptotic cells in Fig. 11A resulting from co-transfections of 293 cells with 1-2µg HA-tagged wild type DRP-1 or various deletion mutants of DRP-1 after 24 hours (average S.D. calculated from triplicates of 100 cells each).

Figures 12A and 12B show by Western analysis that the C-terminal part of DRP-1 is required for its homo-dimerization. In Figure 12A, wild type DRP-1 is shown to undergo specific homo-dimerization. The lanes correspond to the following co-transfections (5µg of DRP-1 constructs and 20µg of RFX1-ΔSmaI constructs/9mm plate): (1) DRP-1-FLAG+RFX1-ΔSmaI-HA (control to rule-out nonspecific attachment of DRP-1 to HA beads or to an irrelevant gene). (2) RFX-ΔSmaI-FLAG+DRP-1-HA(control to rule out nonspecific attachment of DRP-1 to FLAG beads or to an irrelevant gene). (3) DRP-1-FLAG+DRP-1-HA. Both IP directions and their Western blottings are shown. In Figure 12B, truncation of C-terminal 40 amino acids of DRP-1 is shown to abolish its homo-dimerization. The lanes correspond to the following co-transfections (5µg of each construct/90mm plate): (1) DRP-1-FLAG+DRP-1-HA (2) DRP-1-FLAG+DRP-1-Δ40-HA (3) DRP-1-FLAG+DRP-1-Δ73-HA (4) DRP-1-FLAG+DRP-1-Δ85-HA. The lower panel quantitate the immunoprecipitation efficiency of DRP-1-FLAG by the anti-FLAG antibodies.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery by the present inventor of a novel serine/threonine kinase with remarkable homology to the catalytic and CaM-regulatory domains of DAP-kinase. This kinase, named DAP-kinase-related protein 1 (DRP-1), is a 42kDa cytoplasmic protein which exhibits minor associations with insoluble matrix elements.The nucleotide (SEQ ID NO:1) and amino acid (SEQ ID NO:2) sequences of this DRP-1 protein are shown in Fig 1. It is composed of 1742 nucleotides. The predicted initiation and stop codons are boxed, and the polyadenylation signal is underlined. The protein kinase domain is shown in bold letters and corresponds to amino acid residues 13 to 275 of SEQ ID NO:2. This protein displays 80% identity with the catalytic domain of DAP- kinase. The calmodulin-regulatory region is underlined with a dashed line; this region displays high homology to the corresponding region in DAP-kinase. The remainder of the C-terminal short part of the protein (the last 40 amino acid residues corresponding to residues 321 to 360 of

SEQ ID NO:2)differs completely from DAP- kinase. Thus, DAP-kinase-related I protein does
not carry all of the other motifs and protein modules characteristic of DAP- kinase. The
mRNA expression levels transcribed from this gene are low.

5   Another protein, ZIP-kinase, which by virtue of its sequence homology to the
kinase domain of DAP-Kinase, is also a member of the DAP-Kinase-related proteins
subfamily, was recently identified (Kawai et al., 1998; Kogel et al., 1998). Unlike DAP-
Kinase and DRP-1, ZIP-kinase is a nuclear protein, which instead of being regulated by a
calmodulin-binding domain, is activated only by homo-dimerization via its leucine-zipper
motifs (Kawai et al., 1998). To this group of kinases, another two less homologous nuclear
10   proteins, DRAK1 and DRAK2, were recently added (Sanjo et al., 1998). Together they form
a novel subfamily of serine/threonine kinases, as is evident from multiple sequence and
phylogenetic analyses (Inbal et al., 1999). A multiple sequence alignment of the kinase
domain of these serine/threonine kinases is shown in Fig. 2A.

       To check the cellular functions of DRP-1, the laboratory of the present
15   inventor overexpressed wild type DRP-1 in various cell lines and found that it induced
apoptosis as measured by a few parameters. Unlike the wild type DRP-1, a kinase inactive
mutant of DRP-1 (DRP-1 K42A), did not induce apoptosis, although it was expressed at a
similar level in the transfected cells. *In vitro* kinase assays confirmed that DRP-1 K42A is
indeed unable to phosphorylate MLC or under autophosphorylation. Also, a truncated form
20   of DRP-1 which lacks the CaM-regulatory region, could be shown to induce very high levels
of apoptosis, in a similar way to the analogous truncation of the CaM-regulatory region of
DAP-Kinase (ΔCaM; Cohen et al., 1997). Such dependence on the catalytic activity for the
apoptotic function is apparent also in the other members of DAP-kinase-related proteins
(Kawai et al., 1998; Sanjo et al., 1998).

25   In a deletion mutant study, which is also presented in the Example herein, the
existence of a positive element responsible for apoptotic induction which is located at the C-
terminal part of DRP-1 is confirmed. This C-terminal tail of DRP-1 is also essential for its
dimerization. Thus, self-dimerization is a requirement for the functionality of this kinase in
apoptotic assays, although this property can be overrided by a further deletion of the CaM-
30   regulatory region. Like DRP-1, ZIP-kinase-induced cell death is also controlled by its ability
to undergo homo-dimerization via the C-terminal leucine zipper domain (Kawai et al., 1998).
Three point mutations in the leucine zipper domain abolished the homo-dimerization as well as

the ability of ZIP-kinase to undergo autophosphorylation *in vitro* and significantly reduced its ability to induce cell death of NIH 3T3 cells. It seems reasonable to assume that activation of these kinases is achieved by homo-dimerization followed by trans-phosphorylation events.

The high homology in the kinase domains of DAP-kinase and DRP-1, and the finding that they are both localized to the cytoplasm (either in soluble or insoluble forms), imply that they may share the same or closely related substrates. The phosphorylation sites for these kinases on the substrate may be either different or identical. Thus, these kinases may cooperate to induce apoptosis in the same cell type or, alternatively, function independently in different cell types, tissues or organs, or in response to different stimuli or time windows. Another possibility is that these kinases act sequentially along the same signaling pathway to induce apoptosis.

The present invention thus provides for the polypeptide of DRP-1 and for a calmodulin-dependent serine/threonine kinase homologue having the properties of DRP-1, such as the ability to phosphorylate protein in a calcium/calmodulin dependent manner and the ability to induce programmed cell death or apoptosis, and having at least 85% sequence identity to the amino acid sequence SEQ ID NO:2 of DRP-1. Preferably, the calmodulin-dependent serine/threonine kinase homologue has at least 90% sequence identity, and more preferably, at least 95% sequence identity to SEQ ID NO:2.

The term "sequence identity" as used herein means that the amino acid sequences are compared by alignment according to Hanks and Quinn (1991) with a refinement of low homology regions using the Clustal-X program. Such an amino acid alignment is shown in Figs. 2A and 2B where the identical amino acid residues are presented in boxes (cutoff=50%) and homologous amino acid residues, determined according to the PAM 250 matrix, are presented by gray shading (cutoff=65%).

The Clustal-X program referred to in the previous paragraph is the Windows interface for the ClustalW multiple sequence alignment program (Thompson et al., 1994). The Clustal-X program is available over the internet at ftp://ftp-igbmc.u-strasbg.fr/pub/clustalx/. Of course, it should be understood that if this link becomes inactive, those of ordinary skill in the art can find versions of this program at other links using standard internet search techniques without undue experimentation. Unless otherwise specified, the most recent version of any program referred herein, as of the effective filing date of the present application, is the one which is used in order to practice the present invention.

If the above method for determining "sequence identity" is considered to be nonenabled for any reason, then one may determine sequence identity by the following technique. The sequences are aligned using Version 9 of the Genetic Computing Group's GDAP (global alignment program), using the default (BLOSUM62) matrix (values -4 to +11)

5   with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (per each additional consecutive null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the claimed sequence.

In addition to the full length polypeptide of DRP-1 or a functional homologue

10   thereto with at least 85% sequence identity, the present invention also provides for a fragment of the DRP-1 protein of SEQ ID NO:2 which either maintains the ability to induce cell death or lacks this ability but instead is capable of inhibiting the cell killing ability of DRP-1 protein or its functional homologue described above. It was unexpectedly discovered by the present inventor that the 40 amino acid C-terminal tail (residues 321 to 360 of SEQ ID NO:2) is

15   critical to induction of cell death. As the action of DRP-1 is dependent on dimerization, the 40 amino acid tail, by itself, can inhibit the ability of DRP-1 to induce cell death by interfering with and preventing DRP-1 from dimerizing. Furthermore, it was also unexpectedly discovered that the catalytic domain, by itself (without the calmodulin regulatory domain and the 40 amino acid C-terminal tail, e.g., amino acid residues 13 to 275 of SEQ ID NO:2), is

20   super-killing. One of ordinary skill in the art can readily obtain fragments of the full length sequence of the present invention using N-terminal amino peptidases or C-terminal carboxypeptidases. Each fragment can then be readily tested to see if it possesses one of the two functions described herein for such fragments, without undue experimentation.

Besides fragments of DRP-1 having the above-mentioned properties, fragments

25   having an amino acid sequence with at least 85% sequence identity to the above fragments of DRP-1, preferably with at least 90% sequence identity, and more preferably with at least 95% sequence identity, and maintaining the cell death induction or inhibition properties of the original fragment, are also comprehended by the present invention.

Also comphrended by the present invention are chemical derivatives of the

30   DRP-1 and functional homologues and fragments thereof, as defined above, where a "chemical derivative" contains additional chemical moieties not normally part of the DRP-1 amino acid sequence. Covalent modifications of the amino acid sequence are included within

the scope of this invention. Such modifications may be introduced into DRP-1 or fragments thereof by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

5   Cysteinyl residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxylmethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, alpha-bromo- beta-(5-imidazoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl-2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4- nitrophenol, or chloro-7-nitrobenzo-
10   2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

15   Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino acid-containing residues include imidoesters, such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methyliosurea,
20   2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3- butanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the
25   groups of lysine, as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues *per se* has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and e-nitro
30   derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'N-C-N-R') such as 1-cyclohexyl-3-[2-morpholinyl-(4-ethyl)]

carbodiimide or 1- ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

The present invention also comprehends an isolated DNA molecule which includes a nucleotide sequence encoding the DRP-1 protein of SEQ ID NO:2, a functional homologue thereof as described above, or a fragment of DRP-1 which either maintains the ability of DRP-1 to induce cell death or lacks this ability but is instead capable of inhibiting the cell killing ability of DRP-1 protein, as defined above. The isolated DNA molecule according to the present invention is also intended to comprehend a DNA molecule which hybridizes under moderately stringent, preferably highly stringent, conditions to the nucleotide sequence encoding DRP-1 (corresponding to nucleotides 62 to 1141 of SEQ ID NO:1) and which encodes a polypeptide which maintains the cell death induction properties of DRP-1. The present invention further comprehends isolated DNA molecules which hybridize under moderately stringent, preferably highly stringent, conditions to a nucleotide sequence which encodes for a fragment of DRP-1 which either maintains the ability of DRP-1 to induce cell death (i.e., nucleotides 98 to 886 of SEQ ID NO:1 encoding the catalytic kinase domain of DRP-1) or lacks the ability but is instead capable of inhibiting the cell killing ability of DRP-1 protein (i.e., nucleotides 1022 to 1141 of SEQ ID NO:1 encoding the 40 amino acid C-terminal tail of DRP-1). Furthermore, polypeptides encoded by any nucleic acid, such as DNA or RNA, which hybridizes to the nucleotide sequence of nucleotides 62 to 141 of SEQ ID NO:1 under moderately stringent or highly stringent conditions are considered to be within the scope of the present invention as long as the encoded polypeptide maintains the ability of DRP-1 to induce cell death.

As used herein, stringency conditions are a function of the temperature used in the hybridization experiment, the molarity of the monovalent cations and the percentage of formamide in the hybridization solution. To determine the degree of stringency involved with any given set of conditions, one first uses the equation of Meinkoth et al. (1984) for

determining the stability of hybrids of 100% identity expressed as melting temperature Tm of the DNA-DNA hybrid:

$$Tm = 81.5°C + 16.6 (LogM) + 0.41 (\%GC) - 0.61 (\% form) - 500/L$$

where M is the molarity of monovalent cations, %GC is the percentage of G and C nucleotides

5   in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. For each 1° C that the Tm is reduced from that calculated for a 100% identity hybrid, the amount of mismatch permitted is increased by about 1%. Thus, if the Tm used for any given hybridization experiment at the specified salt and formamide concentrations is 10°C below the Tm calculated for a 100% hybrid according to

10   the equation of Meinkoth, hybridization will occur even if there is up to about 10% mismatch.

As used herein, "highly stringent conditions" are those which provide a Tm which is not more than 10°C below the Tm that would exist for a perfect duplex with the target sequence, either as calculated by the above formula or as actually measured. "Moderately stringent conditions" are those which provide a Tm which is not more than 20°C

15   below the Tm that would exist for a perfect duplex with the target sequence, either as calculated by the above formula or as actually measured. Without limitation, examples of highly stringent (5-10°C below the calculated or measured Tm of the hybrid) and moderately stringent (15-20°C below the calculated or measured Tm of the hybrid) conditions use a wash solution of 2 X SSC (standard saline citrate) and 0.5% SDS (sodium dodecyl sulfate) at the

20   appropriate temperature below the calculated Tm of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those which allow less stable hybrids to form along with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash

25   conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE (standard seline-phosphate-EDTA)), 5 X Denhardt's reagent, 0.5% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA at a temperature approximately 20° to 25°C below the Tm. If mixed probes are used, it is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC (Ausubel, 1987, 19989.

30   Additional aspects of the present invention are vectors which carry the isolated DNA molecule according to the present invention and a host cell which is transformed with the isolated DNA according to the present invention.

The present invention further provides for antisense RNA complementary to at least a portion of a messenger RNA (mRNA or "sense" RNA) molecule which is the transcription product of the DNA sequence encoding the DRP-1 protein of SEQ ID NO:2. The antisense DRP-1 sequence can be chemically synthesized or it can be expressed in host cells. However, when expressed in host cells, the expressed antisense RNA must be stable (i.e., does not undergo rapid degradation). Moreover, the antisense DRP-1 RNA, will essentially specifically only hybridize to the sense DRP-1 mRNA and form a stable double-stranded RNA molecule that is essentially non-translatable. In other words, the antisense DRP-1 RNA prevents the expressed sense DRP-1 mRNA from being translated into active DRP-1 protein. When expressed in host cells, a vector-borne antisense DRP-1 sequence may carry either the entire DRP-1 gene sequence or merely a portion thereof as long as the antisense DRP-1 sequence is capable of hybridizing to "sense" DRP-1 mRNA to prevent its translation into DRP-1 protein. Accordingly, an "antisense" sequence of the present invention can be defined as a sequence which is capable of specifically hybridizing to "sense" DRP-1 mRNA to form a non-translatable double-stranded RNA molecule.

The antisense DRP-1 sequence need not hybridize to the entire length of the DRP-1 mRNA. Instead, it may hybridize to selected regions, such as the 5'-untranslated sequence, the coding sequence, or the 3'-untranslated sequence of the "sense" mRNA. In view of the size of the mammalian genome, the antisense DRP-1 sequence is preferably at least 17, more preferably at least 30, base pairs in length. However, shorter sequences may still be useful, i.e., they either fortuitously do not hybridize to other mammalian sequences, or such "cross-hybridization" does not interfere with the metabolism of the cell in a manner and to a degree which prevents the accomplishment of an object of this invention. The greater the length of the antisense sequence and the greater the number of complementary base pairs, the greater the number of non-complementary bases that can be tolerated, especially if the non-complementary bases are scattered. Both the preferred hybridization target on DRP-1 and the preferred antisense sequence length are readily determined by systematic experiment.

Standard methods such as described in Sambrooke et al., (1989) can be used to systematically remove an increasingly larger portion of the antisense DRP-1 sequence from a plasmid vector. Besides the full length antisense DRP-1 sequence, a series of staggered deletions may be generated, preferably at the 5'-end of the antisense DRP-1 sequence. This creates a set of truncated antisense DRP-1 sequences that still remain complementary to

preferably the 5'-end of the sense DRP-1 mRNA and as a result, still forms a RNA molecule

that is double-stranded at the 5'-end of the sense DRP-1 mRNA (complements the 3'-end of an

antisense DRP-1 RNA) and remains non-translatable.

5   The antisense RNA according to the present invention can be used in a method

to neutralize a mRNA molecule, which is the transcription product of the DNA sequence

encoding the DRP-1 protein of SEQ ID NO:2, by allowing the antisense RNA to hybridize to

the DRP-1 mRNA to prevent its translation into DRP-1 protein.

A further aspect of the present invention is directed to a composition, such as a

pharmaceutical composition, which contains DRP-1, functional homologues or fragments

10 thereof and a pharmaceutically-acceptable excipient, carrier, diluent, or auxiliary agent.

An antibody, which specifically recognizes DRP-1 or functional homologues

thereof is part of the present invention as long as the antibody does not cross-react with DAP-

Kinase or ZIP-kinase. For instance, an antibody that specifically recognizes the unique 40

amino acid C-terminal tail of DRP-1, which is not present in DAP-Kinase or ZIP-kinase, is a

15 preferred embodiment of the antibody according to the present invention. Such an antibody

can be used for diagnostic imaging, purification of DRP-1 etc.

The terms "antibody" or "antibodies"as used herein are intended to include

intact antibodies, such as polyclonal antibodies or monoclonal antibodies (mAbs), as well as

proteolytic fragments thereof such as the Fab or $F(ab')_2$ fragments. Furthermore, the DNA

20 encoding the variable region of the antibody can be inserted into other antibodies to produce

chimeric antibodies (see, for example, U.S. Patent 4,816,567) or into T-cell receptors to

produce T-cells with the same broad specificity (Eshhar et al., 1990; Gross, et al., 1989).

Single chain antibodies can also be produced and used. Single chain antibodies can be single

chain composite polypeptides having antigen binding capabilities and comprising a pair of

25 amino acid sequences homologous or analogous to the variable regions of an immunoglobulin

light and heavy chain (linked V_H-V_L or single chain F_V). Both V_H and V_L may copy natural

monoclonal antibody sequences or one or both of the chains may comprise a CDR-FR

construct of the type described in U.S. Patent 5,091,513 (the entire contents of which are

hereby incorporated herein by reference). The separate polypeptides analogous to the variable

30 regions of the light and heavy chains are held together by a polypeptide linker. Methods of

production of such single chain antibodies, particularly where the DNA encoding the

polypeptide structures of the V_H and V_L chains are known, may be accomplished in

accordance with the methods described, for example, in U.S. Patents 4,946,778, 5,091,513
and 5,096,815, the entire contents of each of which are hereby incorporated herein by
reference.

5   As mentioned above, the terms "antibody" or "antibodies" are also meant to
include both intact molecules as well as fragments thereof, such as, for example, Fab and
F(ab')$_2$, which are capable of binding antigen. Fab and F(ab')$_2$ fragments lack the Fc fragment
of intact antibody, clear more rapidly from the circulation, and may have less non-specific
tissue binding than an intact antibody (Wahl et al., 1983). It will be appreciated that Fab and
F(ab')$_2$ and other fragments of the antibodies useful in the present invention may be used for
10   the detection and quantitation of DRP-1 or functional homologues thereof according to the
methods used for intact antibody molecules. Such fragments are typically produced by
proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to
produce F(ab')$_2$ fragments).

               The present invention comprehends not only the intact antibodies or fragments,
15   but also any molecule which includes an antigen binding portion of an antibody such that the
molecule is capable of binding to the antigen. It is well within the skill of the art for the artisan
to make e.g., fusion proteins which include antigen binding portions of an antibody fused to
any other material which is desired to be carried to the antigen binding site, such as marker
molecules, toxins, etc.

20   The antibodies, or fragments of antibodies, of the present invention may be
used to quantitatively or qualitatively detect the presence of DRP-1 or functional homologues
according to the present invention in a sample. The antibody according to the present
invention may also be used for the isolation and purification of DRP-1 or homologues and
fragments thereof, such as in an affinity column where the antibodies are immobilized on a
25   solid phase support or carrier.

               By "solid phase support or carrier" is intended any support capable of binding
antigen or antibodies. Well-known supports, or carriers, include glass, polystyrene,
polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses,
polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to
30   some extent or insoluble for the purposes of the present invention. The support material may
have virtually any possible structural configuration so long as the coupled molecule is capable
of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a

bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

5   One of the ways in which the DRP-1-specific antibody can be detectably labeled is by linking the same to an enzyme and used in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. The detection can be accomplished by

10   colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

          Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it

15   is possible to detect DRP-1 protein through the use of a radioimmunoassay (RIA) (Chard, T., "An Introduction to Radioimmune Assay and Related Techniques" (In: Work, T.S., *et al.*, Laboratory Techniques in Biochemistry in Molecular Biology, North Holland Publishing Company, New York (1978), incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter or a liquid scintillation counter or by

20   autoradiography. Radioactively labeled antibodies or antibody fragments can also be used for their capacity to kill cells bound by such antibodies, or cells in the immediate vicinity which are exposed to the radiation from such antibodies. It is also possible to label the antibody with a fluorescent compound, a chemiluminescent or bioluminescent compound.

          The antibody molecules of the present invention may also be adapted for

25   utilization in an immunometric assay (also known as a "two-site" or "sandwich" assay) which is well know in the art.

          In the present specification, the term "programmed cell death" is used to denote a physiological type of cell death which results from activation of some cellular mechanisms, i.e., death which is controlled by the cell's machinery. Programmed cell death

30   may, for example, be the result of activation of the cell machinery by an external trigger, e.g., a cytokine, which leads to cell death. The term "apoptosis" is also used interchangeably with programmed cell death.

The term "tumor" in the present specification denotes an uncontrolled growing mass of abnormal cells. This term includes both primary tumors, which may be benign or malignant, as well as secondary tumors, or metastases, which have spread to other sites in the body.

5   DRP-1 can be used to inhibit growth and metastasis of tumors. Tumor cells are exposed to a variety of death-inducing signals which, in combination with DAP-kinase-related I, can lead to death of the tumor cells. For example, in the blood stream, invading tumor cells must resist programmed cell death that is induced by interactions with cytotoxic T lymphocytes, natural killer cells, and macrophages, and with the cytokines which these hematopoietic cells secrete (e.g., IFNs, TNF, IL-1β). Tumor cells must also resist the apoptotic cell death induced by nitric oxide anions produced by the endothelial cells, and withstand mechanical shearing forces caused by hemodynamic turbulence. Moreover, during the intravasation or extravasation processes, and during growth in a foreign hostile microenvironment, locally produced inhibitory cytokines (e.g., TGF-β or loss of cell-matrix interactions (e.g., detachment from the basement membranes) also trigger apoptotic cell death.

15   DRP-1 is useful in promoting death of tumor cells. The protein may be administered to patients, in particular, to cancer patients, which administration may cause death of the tumor cells. The protein may be administered *per se*, or may be administered by an expression vector comprising a DNA molecule of the present invention.

20   Because DRP-1 displays 80% identity with the catalytic domain of DAP-kinase and has a region which displays a high homology to the calmodulin-regulatory region of DAP-kinase, it is expected that DRP-1 has enzymatic kinase activity, which is calmodulin-dependent. Thus, DRP-1 has use as an enzyme and may be used, for example, as the enzyme in any *in vitro* enzymatic reaction which requires the presence of a kinase enzyme.

25 Accordingly, DRP-1 can be used *in vitro* to catalyze phosphorylation reactions as a kinase.

          DRP-1 is capable of inducing apoptotic cell death when overexpressed in various cell lines. This ectopic cell death is blocked specifically by the death domain of DAP-kinase, suggesting possible crosstalk between these two kinases. Thus, DRP-1 may also be used for promoting the death of normal or tumor cells and for suppressing the metastatic

30 activity of tumor cells. A particular application of the death-promoting aspect is in therapy of diseases or disorders associated with uncontrolled, pathological cell growth, e.g., cancer (primary tumors and metastasis), psoriasis, autoimmune disease and others. Indeed, it is

expected that the DAP-kinase-related protein I of the present invention and DNA encoding it, may be used in the same manner as disclosed in detail in U.S. applications 08/810,712 and 08/631,097, as well as WO 95/10630.

According to a further aspect of the present invention, referred to herein at
5   times as "the screening aspect", DRP-1 DNA molecules are used in order to screen individuals for predisposition to cancer. In accordance with this aspect the screening is carried out by comparing the sequence of each of the DAP-kinase-related I DNA molecules to each of the respective DAP genes in the individual, or by following RNA and/or protein expression. The absence of a DAP-kinase-related I gene, a partial deletion or any other difference in the
10   sequence that indicates a mutation in an essential region, or the lack of a DRP-1 RNA and/or protein which may result in a loss of function may lead to a predisposition for cancer. For screening, preferably a battery of related DAP and DRP-1 genes maybe used, as well as different antibodies.

In the screening aspect, DAP-kinase related product I molecules may also be
15   used for prognostic purposes. For example, if a tumor cell lacks DRP-1 activity, this may reflect high chances of developing metastasis. In addition, DRP-1 positive cells may be more susceptible to control by chemotherapeutic drugs that work by inducing apoptosis, so that the choice of treatment modalities may be made based upon the DRP-1 state of the cells.

The DAP-kinase-related product can be used to screen individuals for
20   predisposition to cancer. There is provided a method for detecting the absence of a DRP-1 gene, a partial deletion or a mutation (i.e., point mutation, deletion or any other mutation) in the DRP-1 genes of an individual, or the absence of a DRP-1 RNA or protein, comprising probing genomic DNA, cDNA, or RNA from the individual with a DNA probe or a multitude of DNA probes having a complete or partial sequence of the DRP-1 genes, or probing protein
25   extracts with specific antibodies.

A particular application of the screening aspect of this invention is in the screening for individuals having a predisposition to cancer, an absence of the gene, or a detected mutation or deletion indicating that the individual has such a predisposition.

One example of a method in accordance with the screening aspect typically
30   comprises the following steps:

(a)   obtaining a sample of either genomic DNA from cells of the individual or cDNA produced from mRNA of said cells;

(b)   adding one or more DNA probes, each of said probes comprising a

complete or partial sequence of a DRP-1 gene;

(c)   providing conditions for hybridization to determine whether the DRP-1

gene is present or absent, i.e., whether there is a match between the sequence of the DNA

5   probe or probes and a sequence in the DNA of said sample or a mismatch, a mismatch

indicating a deletion or a mutation in the endogenous DNA and a predisposition to cancer in

the tested individual.

Other examples of the screening aspect of the invention are well known to the

skilled artisan and include, but are not limited to, Northern blots, RNase protection assays, and

10   various PCR procedures.

The mutation in the DRP-1 gene, indicating a possible predisposition to cancer,

can also be detected by the aid of appropriate antibodies which are able to distinguish between

a mutated, a non-functional and a normal functional DRP-1 gene product. In addition,

mutations that abolish protein translation or transcription due to promoter inactivation can be

15   detected with the aid of antibodies that are used to react with protein cell extracts. Screening

is also possible with respect to metastases.

Having now generally described the invention, the same will be more readily

understood through reference to the following example which is provided by way of

illustration and is not intended to be limiting of the present invention.

20

EXAMPLE

In this study, the identification and the structure/function analysis of a novel

DAP-kinase-related protein, DRP-1, is described, DRP-1 is a 42kDa Ca^{2+}/CaM-regulated

serine/threonine kinase which shows high degree of homology to DAP (Death Associated

25   Protein)-kinase. The homology spans over the catalytic domain and the calmodulin-regulatory

region, whereas the rest C-terminal part of the protein differs completely from DAP-kinase

and displays no homology to any known protein. The catalytic domain is also homologous to

the recently identified ZIP-kinase and to a lesser extent to the catalytic domains of DRAK1/2,

thus forming together a novel subfamily of serine/threonine kinases. DRP-1 is localized to the

30   cytoplasm as shown by immunostaining and cellular fractionation assays. *In vitro* kinase

assays indicate that wild type DRP-1, but not a kinase inactive mutant, undergoes

autophosphorylation and phosphorylates an external substrate in a Ca^{2+}/CaM-dependent

manner. Ectopically expressed DRP-1 is able to induce apoptosis in various types of cells. Cell killing by DRP-1 is dependent on two features: the intact kinase activity and the presence of C-terminal 40 amino acids shown to be involved in self-dimerization of the kinase. Interestingly, further deletion of the CaM-regulatory region overrided the indispensable role of

5   the C-terminal tail and generated a "super-killer" mutant. Finally, a dominant negative fragment of DAP-kinase encompassing the death domain is a potent blocker of apoptosis induced by DRP-1. This implies a possible functional connection between DAP-kinase and DRP-1. The experiments conducted in this study and the results obtained are presented below.

10

MATERIALS AND METHODS
cDNA cloning and Northern Blot Analysis

A PCR fragment of 364 bp was obtained from a λgtll human spleen cDNA library (Clontech) using primers from the deduced DRP-1 sequence,

15   1047-GGCCGGATGAGGACCTGAGG-1066 (SEQ ID NO:13) and

1411- TCCACACTCCCACCCCAGACTC-1390 (SEQ ID NO:14). To obtain the full length cDNA of DRP-1, the same cDNA library was screened with the radiolabeled PCR product. Positive phage clones were isolated, cDNA was subcloned into a BlueScript vector and analyzed by restriction enzyme mapping and DNA sequencing. A 270 bp 3'-fragment from the

20   full length cDNA of DRP-1 was generated by EcoRI digestion, and used to probe polyA+ RNA prepared by a standard procedure from various cell lines.

In vitro Transcription and Translation Assay

The full length cDNA was used as a template for *in vitro* transcription from the

25   T7 promoter. This RNA was translated in reticulocyte lysate (TNT® T7 Quick Coupled Transcription/Translation System; Promega) by conventional procedures, with [^{35}S] methionine (Amersham) as a labeled precursor. The reaction product was then run on 12% SDS-PAGE gel, followed by sodium salicylate incubation for signal amplification. The gel was dried and exposed to X-ray film.

30

In vitro Kinase Assay

293 cells were transfected by a FLAG-tagged wild type DRP-1, DRP-1 K42A

mutant, or mock-transfected. Cell lysates of 293 transfected cells were prepared as previously

described (Deiss et al., 1995). Immunoprecipitation of DRP-1 or DRP-1 K42A mutant from

150µg total extract was done with 20µl anti-FLAG M2 gel (IBI, Kodak) in 500µl of PLB

5　　supplemented with protease and phosphatase inhibitors for 2h at 4°C. Following three washes

with PLB, the immunoprecipitates were washed once with reaction buffer (50 mM HEPES pH

7.5, 20 mM MgCl$_2$, and 0.1 mg/ml BSA). The proteins bound to the beads were incubated for

15 min at 30°C in 50 µl of reaction buffer containing 15 µCi [γ32p] ATP (3 pmole), 50µM

ATP, 5µg MLC (Sigma), and where indicated, also 1µM bovine calmodulin (Sigma), 0.5 mM

10　CaCl$_2$, or 3mM EGTA in the absence of calmodulin/CaCl$_2$. Protein sample buffer was added

to terminate the reaction, and after boiling, the proteins were analyzed on 11% SDS-PAGE.

The gel was blotted onto a nitrocellulose membrane and ^{32}P- labeled proteins were visualized

by autoradiography.

Immunostaining of Cells

DRP-1 transfected or mock-transfected COS-7 cells were plated on glass

cover-slips (13 mm diam.). After 48 hours, the cells were fixed/permeabilized in 3%

formaldehyde for 5 min, methanol 5 min, acetone 2 min. The cells were blocked in 10% NGS

for 30 min and incubated with anti-FLAG antibodies (dilution 1:100; IBI, Kodak) in 10%

20　NGS for 60 min. Rhodamine-conjugated goat anti-mouse secondary antibodies (dilution

1:200; Jackson Immuno Research Lab.) and the nucleic acid dye, Oligreen (dilution 1:5000;

Molecular Probes), for nuclear staining were then applied. The coverslips were mounted in

Mowiol and observed under fluorescence microscope.

25　## Detergent Extraction Assay

Sub-confluent cultures of COS-7 transfected cells, grown on 9 cm plate, were

washed once with PBS and then with MES buffer (50 mM MES pH 6.8, 2.5 mM EGTA, 2.5

mM MgCl$_2$). The cells were extracted for 3 min with 0.5 ml of 0.5% Triton X-100 in MES

buffer supplemented with protease inhibitors. The supernatant (the soluble fraction- Sol) was

30　collected, centrifuged for 2 min. at 16,000x g at 4°C, and the clear supernatant was then

transferred to new tubes. Two volumes of cold ethanol were added and the tubes were

incubated at -20°C for overnight, centrifuged 10 min. at 16,000x g at 4°C and resuspended in

200μl of 2x protein sample buffer without dye. The detergent insoluble matrix (InSol) remaining on the plate was extracted in 200μl of 2x protein sample buffer, scraped from the plate with rubber policeman and collected into tube. The samples were loaded on 10% SDS-PAGE, 100μg protein extracts were loaded on each lane from the Sol fraction,

5 equivalent volumes of InSol were loaded. Analysis of the proteins was done using monoclonal anti-FLAG antibodies (dilution 1:200; IBI, Kodak).

Cell Lines, Transfections and Apoptotic Assays

All cell lines were grown in DMEM (Biological Industries) supplemented with

10 10% fetal calf serum (Bio-Lab). For transient transfection, 1×10^5 cells per well, were seeded in a 6 well plate a day before transfection. Transfections were done by calcium-phosphate method. For cell death assays by inducing overexpression, a mixture containing 1.5 μg of cell death plasmid (expressing either DRP-1 or ΔCaM DAPk mutant) and 0.5 μg of pEGFP-NI plasmid (Clontech) was used. For cell death protection assays we used a mixture containing

15 1.2 μg of cell death inducing plasmid (either DRP-1 or ΔCaM DAPk mutant), 0.5μg of a plasmid to be tested for cell death protection (expressing DAPk-DD, DN FADD or luciferase as negative control), and 0.5μg of pEGFP-NI plasmid. Cells were counted and photographed 24 hours after transfection. In each transfection, three fields, each consisting of at least 100 GFP-positive cells, were scored for apoptotic cells according to their morphology. When

20 indicated, cell lysates were prepared from the transient transfection at 24 hours, for protein analysis. The transfections of Rat embryo fibroblasts (REF) and FACS analysis of transfected fibroblasts for DNA content distribution were done as previously described (Kissil et al., 1998).

25 ## Co-immunoprecipitation assays

293 cells grown in 90mm plates (1×10^6 cells/plate) were co-transfected with 5μg FLAG-tagged or HA-tagged DRP-1 and 20μg of HA-tagged or FLAG-tagged RFX1-ΔSmaI, respectively, or with DRP-1-HA and DRP-1-FLAG, 5μg each. Immunoprecipitation of DRP-1 or RFX1-ΔSmaI from 1mg total extract was done using anti-FLAG M2 gel or anti-

30 HA as described above. Detection of bound proteins was done using anti-HA antibodies (dilution 1:1000,Babco) or anti-FLAG antibodies. For the deletion mutant study, 5μg of FLAG-tagged fully length DRP-1 were co-transfected with 5μg of HA-tagged DRP-1 deletion

mutants. Immunoprecipitation of DRP-1 from 1mg total extract was done using anti-FLAG M2 gel as described above. Detection of co-immunoprecipitated proteins (the mutants of DRP-1 or full length DRP-1) was done using anti-HA antibodies.

5   Nucleotide sequence accession number

The nucleotide sequence of human DRP-1 has been submitted to the GenBank™/EBI Data Bank (accession no. AF052941). The murine DRP-1 is also deposited at the GenBank™/EBI Data Bank (accession no. AF052942).

10   RESULTS

Cloning of DRP-1

To identify proteins that share homologous sequences with DAP-kinase, EST databases were searched using the BLAST™ program. Two ESTs of human and murine origin showed remarkable amino acid homology to the catalytic domains of DAP-kinase and the recently identified protein ZIP-kinase (79.5% and 80.2% identity, respectively). A second EST search was performed using the 5' and the 3' ends of the human EST, which identified a few more overlapping ESTs. A putative novel cDNA sequence was generated and used to design primers for cloning the full length cDNA. PCR performed on human spleen cDNA library amplified a 364 bp fragment that was further used to screen the same library. The full 20   length cDNA was then isolated, subcloned into BlueScript vector, and sequenced.

The isolated cDNA was found to be 1742 bp long and to contain a serine/threonine kinase domain with all of the 12 characterized subdomains present (Park et al., 1997, Fig. 1A). Sequence alignment indicated that the catalytic domain of DRP-1 has 80%sequence identity to that of DAP-kinase and ZIP-kinase, yet less 50% sequence identity 25   to the newly identified DRAK proteins (Fig. 2A). Like DAP-kinase but unlike ZIP-kinase, DRP-1 carries a typical CaM-regulatory region adjacent to its catalytic domain, as shown in Figures 1 and 2B. As compared with other kinases such as CaKIIa and MLCK, DRP-1 has the highest homology to DAP- kinase in this region, as shown in Figure 2B. The remaining short stretch of amino acids at the C-terminal part of DRP-1 (40 amino acid tail) displays no 30   homology to any known protein.

Expression of DRP-1 and Tissue Distribution

To check the RNA expression of DRP-1, polyA+RNA was prepared from various cell lines and hybridized to a probe designed from the less conserved region of DRP-1. A single weak band of 1.9 kb appeared in some cell lines, in a Northern blot analysis of poly A+RNA (3 micrograms) extracted from various cell lines (Fig. 3A), suggesting that the

5   mRNA is expressed at low amounts in HeLa, 293 and MCF-7 cells. The mRNA was hybridized with a radiolabeled human DRP-1 probe. The position of the transcript is indicated by an arrow. From PCR analysis of various cDNA libraries and the data gathered from EST searches, it was concluded that human DRP-1 is expressed, at least, in spleen, colon, breast, and leukocyte tissue.

10   *In vitro* transcription and translation assays conducted in reticulocyte lysates using the cloned DRP-1 cDNA as a template generated a single protein band of about 42 kDa in size, as predicted by its sequence. This protein band, obtained by Western blot analysis of *in vitro* transcribed and then translated DRP- 1, is shown in Figure 3B. A FLAG-tagged DRP-1 was then cloned into pCDNA3 vector and expressed in HeLa cells. A protein of 42

15   kDa was evident upon immunoblot analysis of the cell lysates with anti-FLAG antibodies, shown in Figure 3C. In this case, 24 hours following transfection, the cells were harvested and lysed. The extracted proteins were separated by SDS-PAGE and then immunoblotted with anti-FLAG antibodies.

20   Cellular Localization of Ectopically Expressed DRP-1

In order to follow the cellular localization of the exogenous DRP-1, FLAG-tagged DRP-1 was expressed in COS-7 cells. COS-7 cells were transfected by a FLAG-tagged DRP-1 cloned in pCDNA3 vector, fixed and permeabilized in 1% formaldehyde followed by methanol/acetone treatment. Cells were visualized under fluorescence

25   microscope. Immunoblot analysis proved that DRP-1 was expressed in these cells. For the immunostaining procedure, the non-transfected (Fig. 4A) and DRP-1 transfected (Fig. 4B) COS-7 cells were then fixed and reacted both with Oligreen for nuclear staining and anti-FLAG antibodies for DRP-1 staining. Specific DRP-1 staining was detected in the cytoplasm of these cells, as shown in Figure 4B.

30   A gentle cell extraction was performed with nonionic detergent, 0.5% TRITON X-100, that removes lipids and soluble proteins, leaving intact the detergent insoluble matrix composed of the nucleus, the cytoskeleton framework, and cytoskeleton-

associated proteins. In contrast to DAP-kinase, which is exclusively localized to the cytoskeleton, and hence found only in detergent insoluble fractions (Cohen et al., 1997, Fig. 4C), DRP-1 was preferentially eluted from the detergent soluble fraction, while a small amount was eluted from the insoluble fraction, as shown in Figure 4C. Thus, it was concluded

5   that DRP-1 is a cytoplasmic protein with minor association with insoluble matrix components.

Intrinsic Kinase Activity of DRP-1

        To test whether DRP-1 functions as a kinase as predicted from the amino acid sequence, an *in vitro* kinase assay was performed using myosin light chain (MLC) as an

10   exogenous substrate. This substrate was chosen because it is phosphorylated by DAP-kinase (Cohen et al., 1997). DRP-1 was transfected into human kidney 293 cells, immunoprecipitated, and incubated with MLC in the presence and absence of Ca2+ and calmodulin. Both MLC phosphorylation and DRP-1 autophosphorylation were evident, as can be seen from Figure 5A.

15   In assaying the *in vitro* kinase activity of DRP-1, the proteins were assayed in the presence or absence of CA2+/CaM and MLC. The proteins were run on 11% SDS-PAGE and blotted to nitrocellulose membrane. Figure 5A shows the autophosphorylation of DRP-1 and MLC phosphorylation, respectively, as seen after exposure of X-ray film. Figure 5B shows the DRP-1 proteins by incubation of the same blot with anti-FLAG antibodies and ECL

20   detection.

        The addition of Ca2+/calmodulin to the reaction mixture increased the amount of phosphorylated MLC, in accordance with the assumption that, like DAP-kinase, DRP-1 is negatively regulated by the autoinhibitory calmodulin binding domain, and that this inhibition is removed by the binding of Ca^{2+}/calmodulin. A catalytically inactive mutant of DRP-1,

25   DRP-1 K42A, did not phosphorylate MLC and failed to undergo autophosphorylation even though higher amounts of DRP-1 protein were present, as can be seen from Figure 5A. Thus, DRP-1 was found to function *in vitro* as a kinase that is capable of phosphorylating itself and an external substrate. This latter property is stimulated by the addition of Ca2+ and calmodulin.

30

DRP-1 Induces Apoptosis in a Variety of Cell Lines

The high homology to DAP-kinase in the kinase and calmodulin-binding regions suggested the value of checking whether DRP-1 is involved in apoptosis. The wild type DRP-1 and the catalytically inactive mutant of DRP-1, DRP-1 K42A, which are cloned in pCDNA3 vector, were transfected into 293 cells. To quantitate the number of apoptotic cells,

5   these constructs were transfected with a vector expressing the GFP protein. The GFP protein was used as a marker to visualize the transfected cells and to assess the apoptotic frequency among the transfectants according to morphological alterations. Apoptotic cells were scored after 24 hours. Overexpression of the DRP-1 resulted in massive apoptotic cell death (50-60%), as compared to the basal level of apoptotic cells caused by transfection of the

10 non-relevant gene luciferase, shown in Figures 6A-6B and 7.

Most of the GFP positive green cells rounded up and shrunk; some of them showed cytoplasmic blebs, and some were further fragmented into "apoptotic bodies." In addition, some of the transfected cells detached from the plate. This apoptotic cell death was only slightly lower than that of an activated DAP- kinase mutant lacking the autoinhibitory

15 calmodulin regulatory region (ΔCaM; apoptotic values of 70-80%). In contrast, when the cells were transfected with the kinase inactive mutant of DRP-1, DRP-1 K42A, as shown in Figures 6A-6D and 7, no apoptosis was observed. This experiment was repeated six times with reproducible results.

Western blot analysis of transfected cells, using anti- FLAG antibodies,

20 confirmed the expression of both the exogenous wild type and K42A mutant of DRP-1 (Figures 8A and 8B). Similar results were also observed in human SV-80 fibroblasts. In another type of assay, the effect of ectopically expressed DRP-1 on the DNA content of rat embryo primary fibroblasts (REF cells) was assessed, as previously described (Kissil et al., 1999). The REFS were co-transfected with DRP-1 and a membrane-bound form of GFP and

25 then after 48 hours subjected to FACS analysis of their DNA content. A fraction of cells displaying a sub-G1 population, indicative of cells containing fragmented DNA, appeared exclusively in the DRP-1 transfected cells but not in cells transfected with a control vector or with the DRP-1 K42A mutant form. No effect was found on cell cycle distribution of the viable cells.

30 To obtain the results shown in Figures 6A-6D, 1×10^5 293 cells/well were co-transfected with FLAG-tagged wild type DRP-1 or K42A mutant of DRP-1, 1.5 microgram/well and GFP, 0.5 microgram/well. GFP positive cells were visualized under

fluorescent microscope and scored for the appearance of apoptotic morphology 24 hours after transfection. Apoptotic cells are indicated by arrows. The fluorescent microscopic images correspond to 293 cells transfected by pCDNA3-luciferase as negative control (Fig. 6A), pCDNA#-deltaCaM DAP-kinase as positive control (Fig. 6B), pCDNA3-DRP-1 (Fig. 6C),

5   pCDNA3-K42A DRP-1 (Fig. 6D).

In Figure 7, graphs show the percentage of apoptotic cells resulting from the above-mentioned transfections (average ± S.D. calculated from triplicates of 100 cells each). The scores were taken from the same experiment shown in Figures 6A-6D.

In Figures 8A and 8B, proteins extracted from the transfected cells were

10 separated on 10% SDS-PAGE and blotted to nitrocellulose membrane. The blot was hybridized with anti-FLAG antibodies for DRP-1 detection and anti-vinculin antibodies to quantitate the loaded protein amounts. The proteins were prepared from the same experiments shown in Figures 6A-6D.

15 DAP Kinase Death Domain Protects From DRP-1 Induced Apoptosis

The structural homology of DRP-1 to DAP-kinase, the common regulation by Ca2+/calmodulin, and the finding that both proteins caused apoptosis upon overexpression, suggested that they function along a common apoptotic pathway. In order to test this possibility, the effect of the dominant-negative DAP- kinase death domain (DAPk DD) on

20 DRP-1-induced cell death was analyzed. The laboratory of the present inventor showed recently that overexpression of the fragment encompassing the death domain of DAP-kinase acts as a specific dominant-negative mutant, negating the effects of the full length protein (Datta et al., 1997). As a consequence, it protected cells from TNF-alpha, Fas and FADD/MORT1-induced cell death (Datta et al., 1997).

25 It has now been discovered that DAPk DD protected cell death induced by DRP-1 in 293 cells. As shown in Figure 9A, the apoptotic ratio dropped from 64.3% to 24.7%. A control transfection including DRP-1 and a non-relevant luciferase DNA excluded the possibility that this blockage was simply due to larger amount of DNA used in the transfection. Moreover, the effect of DAPk DD was specific, since the death domain of

30 FADD failed to manifest a similar effect. (Figure 9A). Western blot analysis of transfected cells using anti-FLAG antibodies confirmed the expression of the exogenous DRP-1 in all transfections, as shown in Figure 9B. This experiment was repeated three times with

reproducible results. The ability of the death domain of DAP- kinase to block death induced

by DRP-1 implies that DAP-kinase and DRP-1 function along a common pathway.

To obtain the results shown in Figure 9A, 1x10^5 cells/well of 293 cells were

co-transfected with 1.2 microgram/well of FLAG-tagged wildtype DRP-1 and 0.5

5   microgram/well of GFP. The scores are the percentage of apoptotic cells given as average ±

S.D. and calculated from triplicates of 100 cells each.

To demonstrate the DRP-1 protein expression in 293 transfected cells shown in

Figure 9B, proteins extracted from the transfected cells were separated on 10% SDS-PAGE

and blotted to nitrocellulose membrane. The blot was hybridized with anti-FLAG antibodies

10   for DRP-1 detection and anti-vinculin antibodies to quantitate the loaded protein amounts.

The proteins were prepared from the same experiment shown in Figure 9A.

Deletion of the C-terminal tail of DRP-1 abolishes its apoptotic activity, while further

truncation of the CaM-regulatory region strongly enhances the apoptotic effect

In order to further understand the mode of DRP-1 action in apoptosis,

constructs containing C-terminal truncations of DRP-1 tagged by HA were constructed (Fig.

10A). DRP-1 Δ40 lacks the most C-terminal part of DRP-1 which displays no homology to

any known protein. DRP-1 Δ73 lacks, in addition to that, the CaM-regulatory region of DRP-

1, and DRP-1 Δ85 contains only the catalytic domain. The wild type DRP-1 and the various

20   truncation mutants of DRP-1 were transfected into 293 cells. Induction of apoptotic cell

death was assayed as mentioned above in DRP-1 induced apoptosis. Overexpression of the

wild type DRP-1 resulted in apoptosis (25%) while the DRP-1 Δ40 had no effect in these

assays. On the other hand, further truncations of the CaM-regulatory region, yielded mutants

(Δ73, Δ85) which acted as "super-killers" (~90% apoptosis) (Figs. 11A and 11B). This

25   experiment was repeated three times with reproducible results. Western blot analysis of

transfected cells, using anti-HA antibodies confirmed the expression of all DRP-1 forms (Fig.

10B). Thus, the dependence of the apoptotic effect of DRP-1 on its kinase activity was

confirmed again, since removal of the CaM-regulatory region which acts as an autoinibitory

domain generates a constitutively active kinase. In addition, the existence of a positive

30   module in the C-terminal region of DRP-1, which is necessary for its pro-apoptotic effect,

provided that the CaM-regulatory effect is still present, is shown.

The C-terminal part of DRP-1 functions as a homo-dimerization domain

Western analysis performed on proteins extracted from 293 cells transfected by FLAG-tagged DRP-1 revealed an additional band (not shown). This observation led the present inventor to test whether DRP-1 can undergo homo-dimerization. To this aim, two constructs expressing DRP-1 fused to either FLAG or HA tags were co-transfected into 293 cells and classical pull-down experiments with each one of the two epitopes were performed. FLAG-tagged DRP-1 could be shown to bind specifically to HA-tagged DRP-1 in both IP directions (Fig. 12A, see lane 3 in both IP Panels). No binding of DRP-1-HA to FLAG beads or to the irrelevant cytoplasmic protein RFX-ΔSmaI could be observed (Fig. 12A, see IP anti-FLAG panel, lanes 2 or 1+2, respectively). Also non-specific binding of DRP-1-FLAG to HA bead or to RFX-ΔSmaI protein could not be detected (Fig. 12A, see IP anti-HA panel, lanes 1 or 1+2, respectively). Western analyses confirmed the expression of all proteins in these cell extracts (Fig. 12A, see Western panels).

The observation that a C-terminal truncation of 40 amino acids in DRP-1 abolished its apoptotic effect upon ectopic expression in 293 cells, prompted the present inventor to test whether this domain may be involved in the homo-dimerization of DRP-1. DRP-1-FLAG was co-expressed in conjugation with the various deletion mutants of DRP-1 tagged by HA. A strong binding of DRP-1-FLAG to the wild type DRP-1-HA was detected, whereas the binding to DRP-1 Δ40 was mostly abolished (Fig. 12B, upper IP panel, compare lane 1 to 2-4). Western analysis confirmed the expression of wild type DRP-1-HA and all other DRP-1-HA deletion mutants in these transfections (Fig. 12B, see Western panel). Lower IP panel confirmed the expression of wild type DRP-1-FLAG in all these transfections. Thus, the present inventor concluded that a region spanning the C-terminal 40 amino acids of DRP-1 is responsible for its homo-dimerization. This homo-dimerization is probably required for the apoptotic effect of DRP-1, since DRP-1-Δ40 has lost the ability to induce apoptosis in 293 cells (Figs. 11A and 11B).

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This

application is intended to cover any variations, uses or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within know or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the

5   scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent application, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited

10   within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the

15   general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed

20   embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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